



Published in final edited form as:

Gastroenterology. 2011 November ; 141(5): 1842–51.e1-10. doi:10.1053/j.gastro.2011.06.064.

Chronic intestinal inflammation induces stress response genes in commensal *Escherichia coli*

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Abstract

BACKGROUND & AIMS—Intestinal microbes induce homeostatic mucosal immune responses, but can also cause inappropriate immune activation in genetically susceptible hosts. While immune responses to bacterial products have been studied extensively, little is known about how intestinal inflammation affects the function of commensal luminal microbes.

METHODS—Microarrays and real-time PCR were used to profile transcriptional changes in luminal bacteria from wild-type (WT) and *IL-10*^{-/-} (KO) mice monoassociated with a non-pathogenic murine *Escherichia coli* isolate (NC101), which causes colitis in gnotobiotic KO mice. Colonic inflammation, innate and adaptive immune responses were measured in WT and KO mice monoassociated with mutant NC101 lacking selected upregulated genes and in KO mice co-colonized with mutant and parental NC101. Intracellular survival of bacteria within primary mouse macrophages and resultant TNF production was measured.

RESULTS—Significant upregulation of the stress response regulon, including the small heat shock proteins IbpA and IbpB that protect *E. coli* from oxidative stress, was observed in bacteria from KO mice with colitis compared to healthy WT controls. In KO mice, *ibpAB* expression

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revision of manuscript for important intellectual content; study supervision

Disclosure Statement: The authors have no conflicts of interest to disclose.

resulted in reduced colonic histologic inflammation, secretion of IL-12/23p40 by colonic explant cultures, serologic reactivity to NC101 antigens, and IFN γ secretion by stimulated mesenteric lymph node cells. Infection of primary macrophages by bacteria expressing *ibpAB* was associated with decreased intracellular survival and attenuated TNF secretion.

CONCLUSIONS—Chronic intestinal inflammation causes functional alterations in gene expression of a commensal gut bacterium. Further studies of this component of the host-microbial dialogue may identify potential novel therapeutic targets to treat inflammatory bowel diseases.

Keywords

Inflammation; Bacteria; Gene Expression

INTRODUCTION

Relative to higher-order, multicellular organisms, microbes are unique in their ability to rapidly evolve with genetic adaptation to environmental conditions. Adaptation of non-pathogenic commensal microbes, such as those residing in the lumen of the gastrointestinal tract, occurs in response to host physiologic conditions. For instance, fucosylated glycans, mucus, and antigen-specific sIgA have been shown to shape luminal bacterial gene expression^{1–3}. Although these studies indicate that the normal intestinal environment influences the composition and function of the gut microbiota, the impact of specific disease states on intestinal microbial communities is only now beginning to be explored.

A prevailing hypothesis is that inflammatory bowel diseases (IBD) are due to a combination of genetic and environmental factors, which predispose to dysregulated immune responses to the commensal intestinal bacteria⁴. Similar to human IBD, the intestinal microbiota is important in the pathogenesis of chronic, immune-mediated experimental colitis⁴. For instance, mice lacking the *IL-10* gene (*IL-10*^{-/-}) are healthy when raised in a germ-free (GF) environment, but rapidly develop chronic, bacterial antigen-specific T-cell-mediated colitis resembling IBD when transferred to specific-pathogen-free (SPF) conditions^{5, 6}. The inflammation in human IBD and experimental colitis is also characterized by increased tissue levels of reactive oxygen (ROS) and nitrogen species^{7, 8}.

While the intestinal microbiome is complex, preferential expansion of subsets of bacteria including *E. coli* is associated with Crohn's disease^{9, 10}. Certain commensal *E. coli* that adhere to and invade epithelial cells have been implicated in the development of ileal Crohn's disease¹¹. Similarly, in animal models, selective colonization (monoassociation) with a fecal murine isolate of non-pathogenic *E. coli* (NC101) for three weeks induces histological evidence of colitis in ex-GF *IL-10*^{-/-} mice but not in wild-type (WT) controls¹². This strain of *E. coli* exhibits many of the characteristics of adherent/invasive *E. coli* isolated from Crohn's disease patients, including persistence within macrophages (unpublished data).

In addition to the expansion of select species of commensal bacteria such as *E. coli*, broad shifts in the composition of the gut microbial communities are also associated with intestinal inflammation in both human IBD and experimental colitis¹³. However, whether these changes are primary or secondary is unknown nor is much known about how pathologic inflammation affects the function of commensal intestinal microbes. We hypothesize that the unique adaptive capabilities of commensal gut microbes allow them to respond to intestinal inflammation by altering gene expression patterns that increase their survival and virulence. Here we provide evidence that a gut commensal strain, *E. coli* NC101, upregulates its stress response regulon in monoassociated *IL-10*^{-/-} mice with colitis compared to healthy controls. Furthermore, we show using deletion mutants that expression

of two components of the *E. coli* stress response regulon, *ibpA* and *ibpB*, is associated with decreased luminal growth, impaired intracellular macrophage survival, and diminished pro-inflammatory host immune responses. These findings suggest that chronic, immune-mediated intestinal inflammation upregulates a pathway in a commensal gut microbe that ultimately proves to be detrimental to bacterial growth and persistence within macrophages and, at the same time, attenuates the pro-inflammatory host response.

METHODS

Bacterial Strains, Lysates, and Growth Curves

The non-pathogenic murine *E. coli* strain designated NC101 was originally isolated from a randomly-chosen colony from the feces of WT mice raised in SPF conditions¹². The *Enterococcus faecalis* (strain OG1RF) was originally obtained from a human oral isolate kindly provided by Dr. Mark Huycke. NC101 Δ *ibpAB* was constructed using λ -*red* recombinase and NC101 *ibpAB*(pGEN-MCS*ibpAB*) was constructed using standard molecular biology techniques (Supplementary Methods). Bacterial lysates were prepared from bacterial cultures grown in either LB or BHI media using mechanical disruption as described elsewhere¹². For growth curves, LB or BHI was inoculated with an overnight bacterial culture and incubated at 37°C. The OD₆₀₀ of the cultures was measured at the indicated time points.

Isolation of Bone Marrow-Derived Macrophages

The murine fibroblast cell line, L929, was used as a source of M-CSF and was kindly provided by Dr. Scott Plevy (UNC Chapel Hill, NC). Conditioned media from L929 cells, was made as previously described¹⁴. Bone marrow-derived macrophages (BMDM) were cultured from femurs and tibias of WT and *IL-10*^{-/-} 129Sv/Ev mice as previously described¹⁵.

Mice

GF *IL-10*^{-/-} and WT control (both on the 129S6/SvEV background) were originally derived in sterile conditions, by hysterectomy at the Gnotobiotic Laboratory (University of Wisconsin Madison). Mice maintained in germ-free conditions at the National Gnotobiotic Rodent Resource Center at UNC-Chapel Hill were monoassociated with bacteria by swabbing the snout and anus (experiments lasting >10 days) or by oral gavage of 1×10^9 CFU (experiments lasting 10 days) with an overnight bacterial culture. Inoculums for dual-association studies were prepared by mixing equal volumes of overnight cultures of NC101 and NC101 Δ *ibpAB* with same OD₆₀₀ immediately prior to administration. Absence of isolator contamination was confirmed by Gram stain and culture of cecal contents on sheep blood agar or BHI plates under aerobic and anaerobic conditions. Animal use protocols were approved by the UNC-Chapel Hill Institutional Animal Care and Use Committees.

RNA Isolation From Cecal Contents

Approximately 300mg of freshly-harvested cecal or transverse colon contents were snap frozen in N₂ (l) and stored at -80°C until ready for use. Frozen samples were thawed into 1mL of RNAProtect Bacteria Reagent (Qiagen) while vortexing, incubated at 25°C for 5 min, and bacterial RNA was isolated as described previously¹⁶.

Microarray Hybridization and Statistical Analysis

RNA samples were analyzed for purity and integrity on Agilent Bioanalyzer chips according to the manufacturer's instructions (Agilent Technologies). All *E. coli* RNA samples were prepared for microarray hybridization according to the expression analysis technical manual

from Affymetrix. Affymetrix *E. coli* Genome 2.0 arrays were washed and stained with the Fluidics Station 450. The arrays were scanned with the GeneChip Scanner 3000 7G Plus with Autoloader. Basic data analysis of the arrays was carried out with MAS 5.0 software to generate intensity values for each gene. Statistical analysis of microarray results was performed using GeneSpring 7.2 software. The list of normalized, differentially-regulated genes was narrowed to those genes with statistically significant differences when grouped by genetic characteristic (Welch t-test, p-value cutoff 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate).

Transcription factor, including initiation factor σ , binding sites were identified in promoter elements of selected *E. coli* genes from the microarray results using the *E. coli* K-12 regulonDB 6.7 online database (<http://regulondb.ccg.unam.mx/index.jsp>)¹⁷.

Real-time PCR

Real-time oligonucleotide primers were designed using Applied Biosystems Primer Express 3.0 software based on *E. coli* MG1655 genomic DNA sequences published in Genbank (Table S4). First-strand cDNA was synthesized from 1 μ g total RNA using M-MLV Reverse Transcriptase (Invitrogen) per the manufacturer's instructions. Two μ L of the reaction were added to 3.75 pmol of each primer and 2 \times SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 12.5 μ L and analyzed in an ABI 7900HT thermocycler using the manufacturer's universal thermocycling conditions: 95°C for 10 minutes then 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The specificity of amplification was confirmed by melting curve analysis and agarose gel electrophoresis of PCR products. All reactions were run in triplicate. Fold-change was calculated using the $\Delta\Delta$ Ct method relative to *E. coli* 16S rRNA.

Quantification of Luminal Bacteria

A small amount of cecal and mid-colonic luminal contents were collected into pre-weighed, sterile tubes. Assuming that 1g of contents has a volume of 1mL, the samples were serially diluted to 10⁻⁷, 10⁻⁸, and 10⁻⁹ in sterile phosphate-buffered saline (PBS). One-hundred μ L of each dilution were plated onto BHI agar and incubated at 37°C for 24 hours under aerobic conditions. Results were expressed as colony forming units (CFU)/g of contents.

Histological Scoring

Sections of colon (proximal, transverse, distal) and cecum, were fixed in 10% neutral buffered formalin for 24–48 hrs. The fixed tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Individual sections were scored for severity of inflammation by a single, blinded individual using a validated scale of 0–4¹². Where indicated, a composite colonic inflammation score was obtained by summing the scores of four individual colonic sections (cecum, proximal, mid, and distal colon) from each animal.

Western Blots

Fifteen μ g/lane of NC101 lysate prepared as described above were resolved on a 10% SDS/polyacrylamide gel, transferred to PVDF membrane, probed with 1:1000 dilution of mouse serum in 5% dry milk-TBST for 90 min, washed 3 times in TBST, incubated with 1:5000 dilution of sheep anti-mouse IgG-HRP in 5% dry milk-TBST for 60 min, washed 3 times, incubated with ECL-Plus (Amersham) for 5 min, and then exposed to X-ray film.

Colonic Tissue Fragment Cultures

Colonic tissue fragment cultures were prepared from the proximal colon as previously described^{5, 12}. Briefly, the colonic tissue fragments were washed with PBS then RPMI

containing 50 μ g/mL gentamicin, blotted dry, and weighed. Fragments were placed into 1mL of complete RPMI in 24-well plates, cut into pieces with scissors, and incubated for 20 hours at 37°C. Supernatants were collected and stored at -20°C.

Mesenteric Lymph Node Preparation and Culture

Mesenteric lymph nodes (MLN) were aseptically removed and single cell suspensions were prepared by gentle disruption between sterile, frosted glass microscope slides. Cells were washed and resuspended in RPMI with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 0.05mM 2-mercaptoethanol, and penicillin (100U/ml)-streptomycin (100 μ g/ml). 5×10^5 cells/well in quadruplicate were stimulated with either media control, or NC101 or *E. faecalis* lysates (5 μ g protein/mL) in 96-well plates (final volume of 0.2mL per well) for 72 hrs¹⁸. Culture supernatants were collected and stored at -20°C prior to cytokine quantification.

Gentamicin Protection Assays

Bacterial uptake and survival within macrophages was measured as described previously¹⁹. Briefly, approximately 10 bacteria/cell were added to BMDMs and incubated for 90 min and then treated for 90 min with media containing 100 μ g/mL gentamicin. Supernatants were removed and stored at -20°C for future cytokine analysis. In separate plates, after the initial gentamicin treatment and washing, 20 μ g gentamicin/mL was added and plates were incubated for an additional 23.5 hrs after which supernatants were removed and stored at -20°C for future cytokine analysis. Survival ratios were calculated by dividing the number of bacteria detected after 24 hrs of gentamicin treatment by the number detected after 1.5 hrs of gentamicin treatment.

Cytokine Measurements

To measure amounts of TNF secreted by infected BMDM, IFN- γ secreted by stimulated MLN cells and IL-12/23 p40 secreted by colon tissue fragment cultures, we used commercially available monoclonal anti-mouse TNF (Cat.No. 555268), IFN- γ (Cat.No. 551866), and IL-12/23 p40 (Cat.No. 555165)- specific capture and detection reagents (BD Biosciences Pharmingen) in enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's protocols with the following exceptions: 1) five washes were performed for all wash steps, and 2) for IL-12/23p40 ELISAs, we followed the TNF ELISA protocol, but used IL-12/23 capture and detection reagents. Cytokine levels were measured in duplicate supernatants and compared to standard curves generated using recombinant murine cytokines per the manufacturer's instructions.

Statistical Analysis

Microarray data were analyzed as described above. All other data are presented as the mean \pm SEM. P values were calculated using the unpaired, 2-tailed Student's T-test.

RESULTS

The Intestinal Inflammatory Milieu Induces Stress Response Genes in a Commensal *E. coli* Strain

While the effects of bacterial products on intestinal immune responses are fairly well-characterized, the impact of inflammation on commensal bacterial function is poorly understood. We hypothesized that luminal bacteria adapt to chronic, immune-mediated colitis by altering the expression of genes in a manner that promotes bacterial survival in a hostile environment. To test this hypothesis, we monoassociated GF WT or *IL-10*^{-/-} mice with the non-pathogenic, commensal murine strain *E. coli* NC101, which was previously

shown to cause a Th1/Th17-mediated colitis in *IL-10*^{-/-}, but not WT, mice²⁰. Consistent with prior results, *IL-10*^{-/-}, but not WT, mice had moderate histologic cecal inflammation 3 and 7 weeks post-colonization (Figure 1A). Similarly, innate immune responses, measured by spontaneous secretion of IL-12/23p40 from colonic explant cultures, were increased in *IL-10*^{-/-} mice compared to WT mice colonized with NC101 (Figure 1B). Together, these data confirm that NC101 induces moderate chronic inflammation in monoassociated *IL-10*^{-/-}, but not WT, mice.

We next performed transcriptional profiling of luminal bacteria isolated from the ceca from colitic *IL-10*^{-/-} and healthy WT mice monoassociated with NC101 for 7 weeks using microarrays. Sixty-four genes were upregulated 1.5-fold and 150 genes were downregulated 2-fold in NC101 from *IL-10*^{-/-} mice with colitis compared to NC101 from WT mice (Tables S1 & S2). In general, genes encoding enzymes of amino group and butanoate metabolism were differentially regulated and TCA cycle-associated genes were downregulated (Table S3). Interestingly, 27% of the identified upregulated genes are involved in *E. coli* stress responses and many of the genes are transcribed by alternative σ factors previously identified to control the *E. coli* stress response regulon (Table 1). Real-time PCR results of selected upregulated genes confirmed the microarray results (Figure S1). These data suggest that *E. coli* NC101 responds to chronic intestinal inflammation, in part, by upregulating stress response genes. For example, acid tolerance genes (*gadAB*, *hdeAB*, *yhiX*, *evgA*) are upregulated although pH of cecal contents is no different (data not shown). Furthermore, some of the most highly upregulated genes (*ibpB*, *ibpA*, *oxyS*) have been shown to protect bacteria from ROS²¹⁻²⁴, which are increased in intestinal tissue of patients with IBD and animals with experimental colitis^{7, 8}.

Expression of Genes Encoding the Small Heat Shock Proteins IbpAB in *E. coli* Decreases Bacterial Survival *in vivo* and Attenuates Experimental Colitis

Assuming bacteria upregulate stress responses during intestinal inflammation to survive, we hypothesized that deletion of specific stress response genes from bacteria may decrease their survival and virulence in the inflammatory milieu resulting in attenuated intestinal inflammation. Specifically, since *ibpA* and *ibpB* (*ibpAB*) are among the most highly upregulated stress response genes and have been shown to protect *E. coli* from ROS, we predicted that these genes enhance the survival and virulence of NC101 in the intestine of *IL-10*^{-/-} mice with colitis. To test this hypothesis, we constructed a mutant strain of NC101 lacking *ibpAB* (NC101 Δ *ibpAB*) and measured growth rates and indicators of chronic, immune-mediated colitis in monoassociated WT and *IL-10*^{-/-} mice. Interestingly, while *in vitro* growth of NC101 and NC101 Δ *ibpAB* was similar, luminal concentrations of NC101 Δ *ibpAB* were significantly greater than NC101 in monoassociated WT and *IL-10*^{-/-} mice (Figure 2). We confirmed the absence of *ibpAB* expression in cecal bacteria from NC101 Δ *ibpAB*-monoassociated *IL-10*^{-/-} mice using real-time PCR (Figure S2). These findings suggest that, contrary to our original hypothesis, expression of *ibpAB* in NC101 limits its growth *in vivo* independent of intestinal inflammation.

To determine whether *in vivo* survival differences between the two strains are associated with differences in their colitogenic potential in *IL-10*^{-/-} mice, we quantified colonic inflammation in the two groups of mice. Increased bacterial density correlated with higher cumulative colitis scores in *IL-10*^{-/-} mice monoassociated with NC101 Δ *ibpAB* compared to NC101 (Figures 3A&B).

Since others have demonstrated that enhanced humoral immune responses to microbial, including *E. coli*, antigens in patients with Crohn's disease correlates with a more aggressive disease course²⁵, we questioned whether increased inflammation in NC101 Δ *ibpAB*-compared to NC101-monoassociated *IL-10*^{-/-} mice was associated with increased levels of

anti-*E. coli* antibodies. Indeed we found increased numbers and intensities of immunoreactive bands on Western blots of NC101 lysate probed with sera from NC101Δ*ibpAB*NC101-monoassociated *IL-10*^{-/-} mice (Figure 3C).

IL-12 and IL-23 are produced by macrophages and dendritic cells when microbial products bind to Toll-like receptors (TLRs), and promote the differentiation or expansion of effector Th1 and Th17 cells, respectively, both of which participate in the development of chronic colitis^{26, 27}. To assess the role of mucosal innate immune responses in potentiating colitis in NC101Δ*ibpAB*-monoassociated *IL-10*^{-/-} mice, we measured spontaneous secretion of IL-12/23p40 in colonic explant cultures. Consistent with increased histologic inflammation, colonic explants from NC101Δ*ibpAB*-monoassociated *IL-10*^{-/-} mice secreted more IL-12/23p40 than those from NC101-monoassociated mice suggesting activation of innate immune pathways (Figure 3D).

Since IL-12/23p40 effectively links the innate and the adaptive immune systems, we wanted to determine whether the increased production of IL-12/23p40 observed in NC101Δ*ibpAB*-monoassociated mice is associated with enhanced bacterial antigen-specific adaptive immune responses in these mice. To test this, we measured IFN- γ production by NC101 lysate-stimulated mesenteric lymph node (MLN) cells obtained from NC101Δ*ibpAB*- and NC101-monoassociated WT and *IL-10*^{-/-} mice. Stimulated MLN cells from *IL-10*^{-/-} mice colonized with NC101Δ*ibpAB* secreted more IFN- γ compared to those from mice colonized with NC101 for both 3 and 5 weeks (Figure 3E). There were no significant differences in IFN- γ secretion by MLN cells from the two groups of animals when stimulated with lysates from an unrelated Gram positive bacterium, *Enterococcus faecalis*, and the amounts of secreted IFN- γ were negligible (data not shown). Importantly, in *IL-10*^{-/-} mice monoassociated with a complemented strain of NC101Δ*ibpAB* [NC101Δ*ibpAB*(pGEN-MCS*ibpAB*)] for 3 weeks, luminal bacterial concentrations, histologic inflammation, and IFN- γ secretion by stimulated MLN cells were similar to results obtained in NC101-monoassociated *IL-10*^{-/-} mice (Figure S3). Expression of *ibpAB* in luminal *E. coli* from the NC101Δ*ibpAB*(pGEN-MCS*ibpAB*)-monoassociated mice was confirmed by real-time PCR (Figure S2). Taken together, these results suggest that, contrary to our original hypothesis, expression of *ibpAB* in *E. coli* is detrimental to luminal bacterial survival and attenuates innate, and adaptive mucosal immune responses in both *IL-10*^{-/-} and WT mice. However, the mechanisms by which this occurs is unclear.

Decreased *E. coli* Survival Associated with *ibpAB* Expression Does Not Correlate with Reduced Histologic Inflammation at Early Time Points

Since our initial observations indicated that *ibpAB* expression in NC101 is associated with decreased bacterial growth and attenuated mucosal inflammation at later time points (3 weeks), we hypothesized that reduced mucosal inflammatory responses are due to impaired luminal bacterial survival in NC101-monoassociated *IL-10*^{-/-} mice rather than a direct effect of *ibpAB* on the host immune system. To test this, we dual-associated *IL-10*^{-/-} mice with equivalent numbers of NC101 and NC101Δ*ibpAB* to determine whether host inflammatory responses correlated with the proportion of luminal NC101Δ*ibpAB* or total bacteria. At 5 weeks, there were similar numbers of luminal bacteria in NC101Δ*ibpAB*-monoassociated and dual-associated mice, and 82 ± 4.6% of cecal bacteria in dual-associated mice were NC101Δ*ibpAB* (Figure S4). Histologic inflammation and IL-12/23p40 secretion from colonic explants were equivalent in the two groups of mice suggesting that host inflammatory responses correlate better with total bacteria number than with proportion of *ibpAB*-expressing bacteria (Figure S4). However, since the vast majority of bacteria in the dual-associated mice were NC101Δ*ibpAB*, we cannot exclude the possibility that *ibpAB* expression may directly influence host immune responses independent of luminal bacteria numbers.

Next, we monoassociated *IL-10*^{-/-} mice with NC101 or NC101Δ*ibpAB* for shorter times (3 and 10 days) to determine whether differences in luminal bacterial concentrations at these early time points correlate with differences in the degree of mucosal inflammation as they had at later time points. NC101Δ*ibpAB* grew to higher densities than NC101 in cecal and transverse colon contents as early as 3 days after colonization, and this difference persisted through five weeks in cecal contents, but decreased in magnitude in transverse colon contents (Figures 4A and B). However, at 3 days, the difference in IL-12/23p40 secretion from colonic explants, a measure of early inflammatory responses, and IFN- γ production by NC101 lysate-stimulated MLN cells from the two groups of animals were still relatively small, but statistically significant (Figures 4C & D). Moreover, there was no difference in histologic inflammation at 3 and 10 days (Figure 4E). Thus, peak differences in luminal bacterial concentrations between NC101 and NC101Δ*ibpAB* occurred soon after monoassociation, at a time when differences in measures of inflammation were minimal. Though changes in inflammation may lag alterations in luminal bacterial concentrations, these data suggest that the disparity in inflammation noted between NC101- and NC101Δ*ibpAB*-monoassociated *IL-10*^{-/-} mice may not be entirely dependent on differences in luminal bacterial concentrations of the two strains.

***ibpAB* Expression is Associated with Impaired Survival of *E. coli* NC101 in Murine Macrophages**

Since decreased growth of luminal NC101 compared to NC101Δ*ibpAB* did not completely correlate with decreased measures of inflammation, we wanted to determine whether *ibpAB* expression affected bacterial clearance by innate immune cells. Others have shown that adherent, invasive *E. coli* strains isolated from CD patients survive and replicate within the phagolysosome of murine macrophages and that intracellular persistence in macrophages is associated with constitutive secretion of TNF- α , a pro-inflammatory cytokine important in the pathogenesis of CD²⁸. We therefore hypothesized that *ibpAB* expression may reduce intestinal inflammation in part by decreasing survival of NC101 within mucosal macrophages resulting in decreased TNF secretion. To test this hypothesis, we performed gentamicin protection assays using murine bone marrow-derived macrophages (BMDM) to determine whether NC101 was more vulnerable to killing within macrophages compared to NC101Δ*ibpAB*, and if so, whether this correlated with decreased production of TNF by macrophages. We found that expression of *ibpAB* influenced initial uptake of NC101 in *IL-10*^{-/-}, but not WT, macrophages by reducing the number of internalized bacteria at 1 hr (Figure 5A). However, once internalized, NC101Δ*ibpAB* survived better in macrophages from both mouse backgrounds at 24 hrs than did NC101 (Figure 5A & B). Furthermore, while there was no significant difference in the survival ratio of NC101Δ*ibpAB* between WT and *IL-10*^{-/-} macrophages, NC101 had a significantly lower survival ratio in *IL-10*^{-/-} versus WT macrophages. These data indicate that *ibpAB* expression impairs intracellular survival of NC101 in macrophages, and that this effect is more pronounced in the absence of IL-10. In addition, while NC101-infected WT and *IL-10*^{-/-} macrophages secreted more TNF during the first 3 hours of infection, they produced significantly less TNF from 3–24 hours compared to NC101Δ*ibpAB*-infected macrophages (Figure 5C). Thus, decreased long-term intracellular survival of NC101 in macrophages compared to NC101Δ*ibpAB* correlates with reduced secretion of TNF at later time-points *in vitro*. Whether this effect occurs in intestinal lamina propria macrophages *in vivo* and contributes to the decreased histologic inflammation noted in NC101-monoassociated *IL-10*^{-/-} mice remains to be determined.

DISCUSSION

To the best of our knowledge, this is the first published study of commensal bacterial gene regulation in response to chronic, T-cell-mediated intestinal inflammation. We demonstrated

that the *E. coli* stress response regulon, including the small heat shock proteins IbpAB, is upregulated in the *IL-10*^{-/-} mouse model of IBD. Contrary to our initial hypothesis, *ibpAB* expression inhibits NC101 growth within the intestinal lumen and survival within primary macrophages. This is evident from our studies using *ibpAB*-deficient *E. coli* NC101, which showed enhanced luminal bacterial growth and inflammatory host responses *in vivo*, the ability of *ibpAB*-deficient *E. coli* NC101 to outcompete parental NC101 in dual-association studies, and increased bacterial survival within macrophages and elevated TNF secretion *in vitro*. The reasons why bacteria would upregulate genes in response to inflammation that decrease their survival *in vivo* are currently unclear.

The paradox could be explained by limitations of our experimental model. First, we are studying *E. coli* in an un-natural, monoassociated system without the influences of other commensal microbes, which could affect *E. coli* gene responses to intestinal inflammation. Second, we have only studied gene expression levels in luminal bacteria whereas the results from mucosally-associated bacteria may be quite different. Third, we inferred that *ibpAB* expression in luminal *E. coli* is detrimental to the bacterium because its complete absence resulted in increased growth. However, there could be a range of *ibpAB* expression levels, below which fitness of the bacterium is inversely proportional to expression and above which fitness is proportional to expression. Fourth, the complete absence of IbpAB may cause alterations in expression of other genes, which in turn results in the observed phenotype. However, we detected no differences in mRNA levels of genes adjacent to *ibpAB* in NC101Δ*ibpAB* compared to NC101 (data not shown).

While the functions of *ibpAB* in cultured bacteria are beginning to be elucidated, little is known about their role in *E. coli* within the intestinal lumen. Others have shown that IbpAB promote bacterial survival *in vitro* by functioning as molecular chaperones in conditions of heat shock, enhancing resistance to oxidative stress, and facilitating biofilm formation^{23, 29, 30}. For example, oxidized proteins accumulate in *ibpAB*-deficient *E. coli* exposed to copper ions, suggesting that *ibpAB* protects proteins from copper-induced oxidative damage²³. However, based on our results, we propose that *E. coli ibpAB* function as negative survival factors in the colon, though the mechanisms by which this occurs are unclear. We speculate that IbpAB preserve the function of proteins in certain metabolic pathways that are less-efficient at extracting energy from dietary substances. This is supported by evidence that *ibpAB* can protect metabolic enzymes from oxidative damage²².

Although we have shown that *ibpAB* are upregulated in luminal *E. coli* isolated from animals with experimental colitis compared to healthy controls, the host-derived stimuli and their cognate bacterial pathways that enhance *ibpAB* expression are unknown. *In vitro* studies suggest that both *ibpA* and *ibpB* can be transcribed as an operon from a common promoter that binds the alternative σ factor, σ^{32} , whereas *ibpB* also can be independently transcribed from a separate promoter located between the two genes, which binds σ^{54} , a different alternative σ factor,³¹. Because we noted that both *ibpA* and *ibpB* were upregulated during intestinal inflammation, the major stimulus is probably acting via σ^{32} . However, since the core temperature of the mice is probably minimally changed by colitis (though we did not measure this), we doubt heat is causing increased *ibpAB* expression. More likely, the inflamed intestine produces factors, such as reactive oxygen species, which cause unfolded proteins to accumulate in the cytoplasm of luminal bacteria resulting in upregulation of σ^{32} -dependent *ibpAB* expression. Indeed, others have shown that reactive oxygen species are increased in intestinal inflammation and that *ibpAB* protects *E. coli* from oxidative damage^{7, 8, 21}. Additional studies to identify the factors in the inflamed intestine that induce *ibpAB* in luminal *E. coli* are needed.

In summary, we show that a commensal *E. coli* strain upregulates a broad set of stress response genes, particularly *ibpAB*, in response to intestinal inflammation in monoassociated *IL-10^{-/-}* mice. Furthermore, contrary to our original hypothesis, we provide evidence that *ibpAB* expression in *E. coli* is associated with reduced survival of luminal and intra-macrophage bacteria and increased host pro-inflammatory immune responses. These data suggest that commensal intestinal microbes are not passive bystanders during intestinal inflammation, but dynamically react to host-derived factors, even if it ultimately proves detrimental to bacterial survival in the gut. Further studies of this important host-microbial dialogue may provide clues to the pathogenesis of human IBD and potentially offer novel therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Virginia Miller for her expert advice and critical review of the manuscript and Drs. M. Chelsea Lane and Kim Walker for their technical help creating the mutant and complemented strains of *E. coli* NC101. We acknowledge Dr. Michael Vernon at the UNC Functional Genomics Core for performing the microarray experiments, and the Gnotobiotic, Histology, and Immunotechnology Cores at the UNC Center for Gastrointestinal Biology and Disease (supported by NIH P30DK34987). This work was funded by grants from the UNC Holderness Distinguished Medical Scholars Program (L.G.P.), NIH K08DK087896 (J.J.H.), Crohn's and Colitis Foundation of America (CCFA) Career Development Award (J.J.H.), Center for Gastrointestinal Biology and Disease Pilot Feasibility Grant to J.J.H. (NIH P30DK34987, Robert S. Sandler, PI), NIH R01DK53347(RBS), NIH P40RR18803(RBS), CCFA Gnotobiotic Animal Facility(RBS), and NIH/NCI 1U54CA121852-01A1 National Center for the Multiscale Analysis of Genomic and Cellular Networks(Y.A.L.).

Abbreviations

BHI	brain heart infusion
BMDM	bone marrow-derived macrophages
CD	Crohn's disease
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GF	germ free
IBD	inflammatory bowel diseases
LB	Luria-Bertani
MLN	mesenteric lymph node
PCR	polymerase chain reaction
SPF	specific pathogen free
TBS	Tris-buffered saline
WT	wild-type

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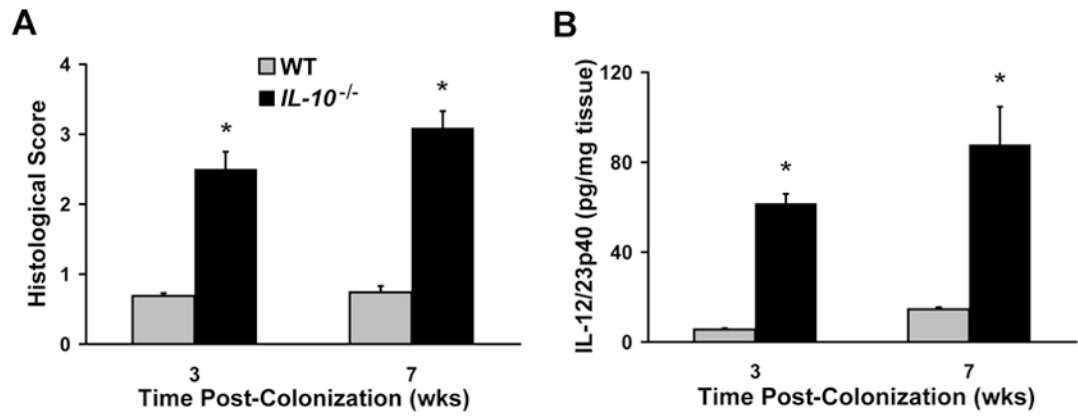


Figure 1.

Spontaneous intestinal inflammation develops in *E. coli* NC101-monoassociated *IL-10*^{-/-} mice. WT and *IL-10*^{-/-} mice were monoassociated with *E. coli* NC101 for 3 and 7 weeks. Histological inflammation in the cecum (A) and spontaneous secretion of IL-12/23p40 from proximal colon explant cultures (B) was measured. Data are means + SEM, n=5 mice/group, *p<0.05 relative to WT.

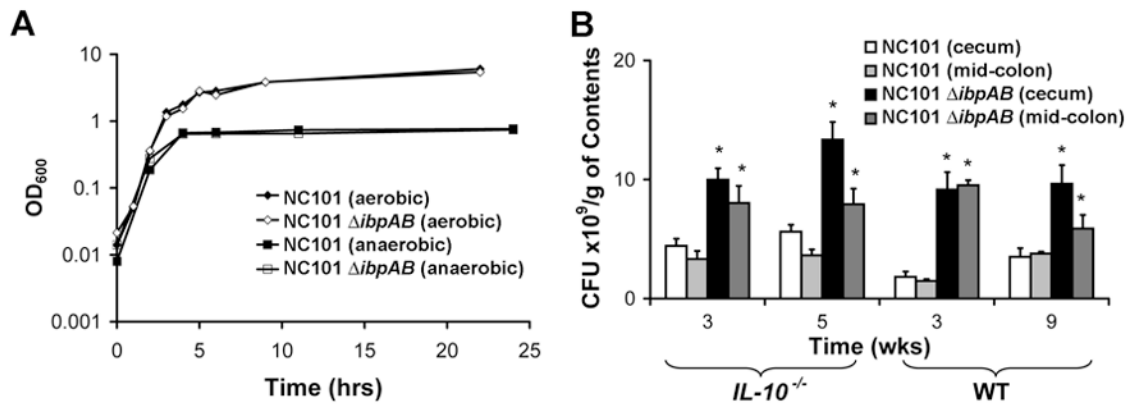


Figure 2. Survival of NC101 is attenuated relative to NC101 $\Delta ibpAB$ *in vivo*, but not *in vitro*. (A) NC101 or NC101 $\Delta ibpAB$ were grown in LB under aerobic or anaerobic conditions and bacterial density was measured using OD₆₀₀. Data are presented as the mean \pm SEM, n=3 cultures/strain. (B) Luminal bacterial concentrations in monoassociated WT and $IL-10^{-/-}$ mice. Data are means + SEM, n=5–6 mice/group, *p<0.05 relative to NC101-monoassociated mice.

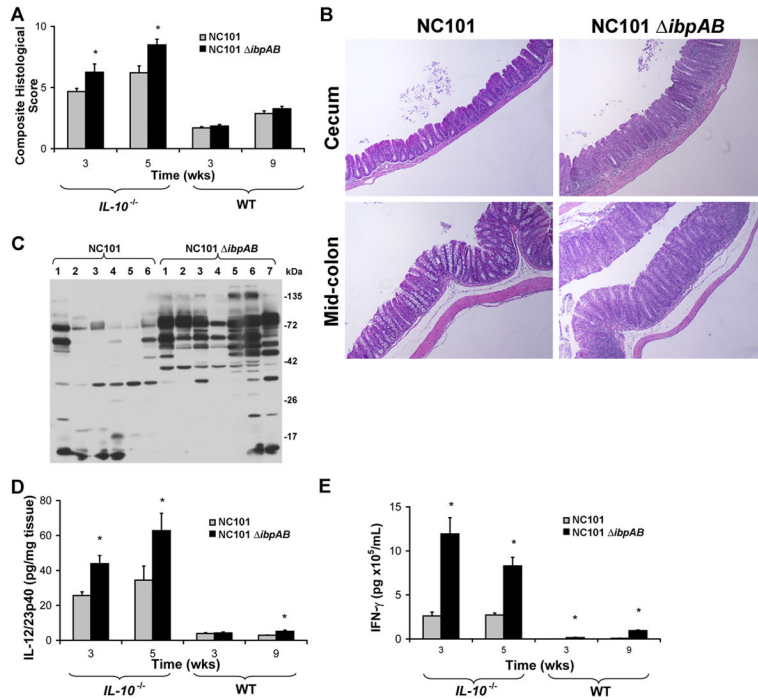


Figure 3. Expression of *E. coli* *ibpAB* is associated with decreased inflammatory responses in NC101-monoassociated *IL-10*^{-/-} and WT mice. (A) Composite blinded histological inflammation scores of 4 colonic segments from *IL-10*^{-/-} or WT mice. (B) Representative photomicrographs of cecum and mid-colon from *IL-10*^{-/-} mice monoassociated for 5 weeks, 20x magnification. (C) Western blots of NC101 lysate using sera from 13 *IL-10*^{-/-} mice monoassociated with the indicated bacteria for 5 weeks. (D) Spontaneous secretion of IL-12/23p40 in culture supernatants from proximal colon explants. (E) IFN- γ secretion by unseparated MLN cells stimulated *ex vivo* with NC101 lysate. Data are means + SEM, n=5–6 mice/group, *p<0.05 relative to NC101-monoassociated mice.

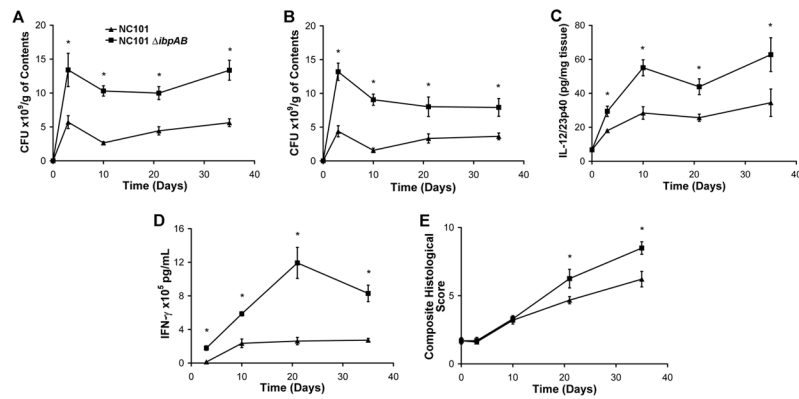


Figure 4. Attenuated pro-inflammatory responses in *IL-10*^{-/-} mice monoassociated with NC101 compared to NC101 Δ ibpAB are not proportional to differences in luminal bacterial concentrations at early time points. Luminal bacterial concentrations in the cecum (A) or mid-colon (B) from *IL-10*^{-/-} mice monoassociated with NC101 or NC101 Δ ibpAB. (C) Spontaneous IL-12/23p40 secretion in proximal colon explant cultures. (D) IFN- γ secretion by unseparated MLN cells stimulated *ex vivo* with NC101 lysate. (E) Blinded composite histological inflammation scores of 4 colonic segments. Data are means \pm SEM, n=5–6 mice/group, *p<0.05 relative to NC101-monoassociated mice.

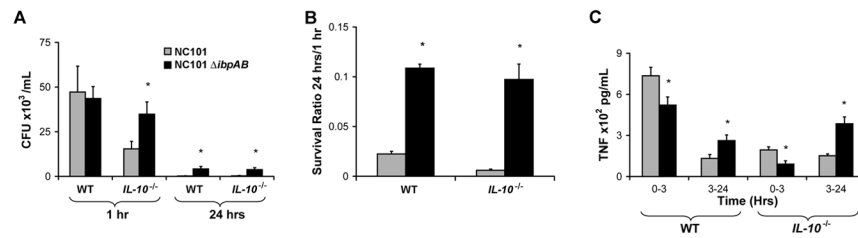


Figure 5. Expression of *ibpAB* by *E. coli* NC101 is associated with attenuated survival within macrophages and decreased TNF secretion. (A) Intracellular survival of NC101 and NC101 Δ *ibpAB* in WT and *IL-10*^{-/-} bone marrow-derived macrophages. (B) Survival ratios (24h/1h) of intracellular bacteria. (C) TNF secretion by NC101- or NC101 Δ *ibpAB*-infected macrophages. Data are means + SEM, 3 wells/condition, representative of results from 3 different mice, *p<0.05 relative to NC101.

Table 1Selected genes upregulated in *E. coli* NC101 during experimental colitis.

Gene	Fold Increase	Description	Alternative σ
<i>ibpB</i>	7.3	Heat shock protein	32,54
<i>glpA</i>	6.9	Glycerol-3-phosphate dehydrogenase, subunit A	
<i>cspH</i>	6.2	Cold shock-like protein	
<i>glpC</i>	5.3	Glycerol-3-phosphate dehydrogenase, subunit C	
<i>gadA</i>	5.1	Glutamate decarboxylase beta, acid tolerance	38
<i>ibpA</i>	4.7	Heat shock protein	32
<i>gadB</i>	4.4	Glutamate decarboxylase beta, acid tolerance	38
<i>oxyS</i>	4.4	Global regulatory RNA, oxidative damage	
<i>ycfR</i>	4.0	Hypothetical protein, biofilm formation	
<i>ydgF</i>	3.6	Hypothetical protein, putative chaperone	
<i>hdeB</i>	3.2	Hypothetical protein, acid resistance chaperone	38
<i>glpD</i>	3.1	Glycerol-3-phosphate dehydrogenase, subunit D	
<i>evgA</i>	2.6	Positive transcription regulator of <i>gadE</i>	
<i>yhiX</i>	2.3	Transcriptional regulator of <i>gadE</i> , <i>gadB</i>	38
<i>inaA</i>	1.8	pH-inducible protein involved in stress response	
<i>rpoH</i>	1.6	RNA polymerase sigma-32 factor	38,54
<i>hslV</i>	1.5	ATP-dependent protease; heat shock protein	32